American Journal of Pathology, Vol. 168, No. 2, February 2006

DOI: 10.2353/ajpath.2006.050407

Stem Cells, Tissue Engineering and Hematopoietic Elements

Canine Cranial Reconstruction Using Autologous Bone Marrow Stromal Cells

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Limited-sized transplants of culture-expanded autologous or allogeneic bone marrow stromal cells (BMSCs) form cortico-cancellous bone in rodent models. Initiation of clinical studies using autologous BMSC transplantation requires effective bone formation among sizable transplants in a large animal model as well as noninvasive techniques for evaluating transplant success. Here, we obtained bone marrow from the femurs of six dogs and expanded BMSCs in tissue culture. Autologous BMSC-hydroxyapatite/tricalcium phosphate (HA/TCP) transplants were introduced into critical-sized calvarial defects and contralateral control skull defects received HA/TCP vehicle alone. At intervals ranging from 2 to 20 months, transplants were biopsied or harvested for histological and mechanical analysis. Noninvasive studies, including quantitative computed tomography scans and ultrasound, were simultaneously obtained. In all animals, BMSC-containing transplants formed significantly more bone than their control counterparts. BMSC-associated bone possessed mechanical properties similar to the adjacent normal bone, confirmed by both ultrasound and *ex vivo* analysis. Evaluation by quantitative computed tomography confirmed that the extent of bone formation demonstrated by histology could be discerned through noninvasive means. These results show that autologous cultured BMSC transplantation is a feasible therapy in clinical-sized bone defects and that such transplants can be assessed noninvasively, suggesting that this technique has potential for use in patients with certain bone defects. *(Am J Pathol 2006, 168:542–550; DOI: 10.2353/ajpath.2006.050407)*

Friedenstein and Owen[1,2] demonstrated the presence of a population of bone marrow-derived stromal cells (BMSCs) with a pluripotent capability. These cells could be distinguished from the majority of the hematopoietic elements in the marrow by their high adherence to the substrate plastic in tissue culture flasks and by a number of morphological, histochemical, and biochemical characteristics. Phenotypically, BMSCs have been found to be capable of differentiating into bone, cartilage, muscle, adipose, and neural tissue elements.[1–6] Populations of BMSCs that include osteoprogenitor cells have been expanded in tissue culture and transplanted into recipient animals. Such heterotopic transplants, whose donor BMSCs have been isolated from species ranging from rodents to humans, form bone in immunodeficient and immunologically intact mouse recipients.[1,7–13] BMSCs have also been shown to repair induced bone defects in various animal models.[14,15]

Successful repair of calvarial defects in patients, whether arising from trauma, tumor resection, or congenital disorders, continues to be a major concern to reconstructive surgeons. The transplantation of bone autograft is the preferred method for reconstructing these areas when they are limited in size. When the volume of the defect is sizable, however, autograft often proves insuf-

Supported by the University of California–San Francisco Research Evaluation and Allocation Committee and the National Institutes of Health (Intramural Research Program of the National Institute of Dental and Craniofacial Research, DHHS; and grant no. 5R01 DE015633 to R.K.N. and R.O.R.).

Accepted for publication October 4, 2005.

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ficient. Surgeons rely on bone graft extenders or alternatives, including osteoconductive matrices such as Interpore and MedPore. Unfortunately, these matrices experience only limited tissue incorporation and can suffer from significant rates of fracture, migration, and infection. As a result, a need exists for a technique for creating new bone for patients with sizable defects.

Our group has previously demonstrated the feasibility of closing calvarial defects in mice with isogeneic mouse BMSCs in conjunction with collagen matrices[15] or with human BMSCs in conjunction with hydroxyapatite/tricalcium phosphate (HA/TCP) particles.[16] However, no study has yet demonstrated the practicality of creating such BMSC transplants as large as those needed clinically. In this study we attempted to close calvarial defects in a large animal model using autologous BMSCs. The aims of this study were to: 1) demonstrate the feasibility of closing critical-size calvarial defects of a size comparable to those seen in patients; 2) demonstrate the practicality of scaling up cell processing and transplant preparation procedures; 3) create large enough transplants to complete noninvasive evaluations of new bone formation; and 4) create large enough transplants to complete biomechanical studies. BMSCs were harvested from healthy dogs, expanded in tissue culture, attached to HA/TCP particles, and autotransplanted into critical-sized calvarial defects. The radiographical, histological, and mechanical characteristics of the transplants were evaluated.

Materials and Methods

Transplant Preparation and Placement

Bone marrow was harvested from the distal femur of six 9-month-old male mongrel dogs, in accordance with an approved NIH animal protocol (97-031). Multicolony-derived strains of BMSCs were obtained from the bone marrow in a manner previously described.[17] Briefly, bone marrow cells were cultured in growth medium consisting of α-minimal essential medium (Invitrogen, Grand Island, NY), 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (Biofluids, Rockville, MD), 10^{-8} mol/L dexamethasone (Sigma, St. Louis, MO), 10^{-4} mol/L L-ascorbic acid phosphate magnesium salt n-hydrate (Wako, Osaka, Japan), and 20% fetal bovine serum of a preselected lot (Equitech-Bio, Kerrville, TX). Cells were cultured at 37°C in an atmosphere of 100% humidity and 5% CO_2.

Trypsin-released cells from passages three or four were pipetted into 50-ml polypropylene Falcon tubes (Becton, Dickinson, and Co., Franklin Lakes, NJ). Separately, HA/TCP particles (Zimmer, Inc., Warsaw, IN) of size range 0.5 to 1.0 mm were isolated using a sieve shaker (CSC Scientific, Fairfax, VA). Between 50×10^6 and 70×10^6 BMSCs were allowed to attach to each gram of HA/TCP particles. Control transplants consisted of HA/TCP particles that were moistened with growth medium but received no cells. The BMSC-free transplants were incubated with the same growth medium (including 20% fetal bovine serum) and for the same length of time (90 minutes) as that used for the BMSC transplants. The mixtures were incubated for 90 minutes at 37°C on a slowly rotating platform. After centrifugation of the mixtures at 1200 rpm for 60 seconds, the supernatant was discarded.

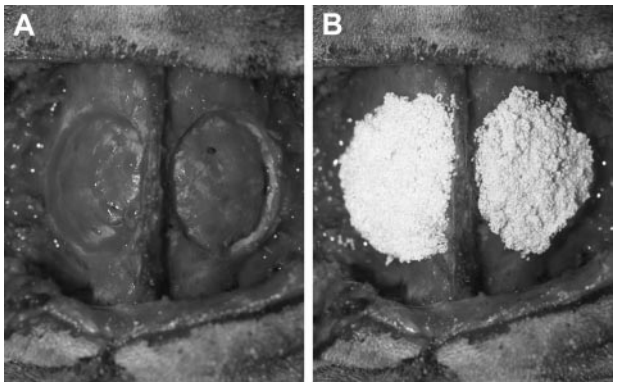

Figure 1. A: The dog calvaria prepared by creating equivalent, bilateral 35-mm bone defects. **B:** A BMSC-HA/TCP transplant was placed in one defect and a control, BMSC-free transplant placed in the other.

Each of the six dogs underwent creation of critical-sized bilateral fronto-parietal cranial defects via a bicoronal scalp incision. Care was taken to elevate the periosteum for a distance of at least 1 cm from the defect. Each defect measured 35 mm in diameter, purposely chosen to be larger than the critical-size defect of 20 mm.[18] In each dog, one defect was filled the BMSC + HA/TCP mixture and the contralateral defect was filled with control HA/TCP particles (Figure 1). Approximately 2 g of the appropriate HA/TCP mixture was needed for each defect.

Between 2 to 3 months after transplantation, a subset of three dogs underwent biopsies of each of its two calvarial transplants. Demineralized tissues were processed to produce hematoxylin and eosin-stained sections that were examined histologically; the extent of bone within each transplant was scored on a semiquantitative, logarithmic scale by blinded observers (Table 1). The extent of bone union between calvarial margin and

Table 1. Semiquantitative Scale for the Estimation of Bone Formation

Score	Extent of bone present within the transplant
0	No bone evident
1	Minimal bone evident (one trabecula)
2	Weak bone formation, occupying only a small portion of the section
3	Moderate bone formation, occupying a significant portion but less than one-half of the section
4	Abundant bone formation, occupying greater than one half of the section

the transplant was analyzed using histomorphometric techniques.

All six dogs underwent sacrifice and full harvest of the calvaria at time intervals of 6 to 20 months after transplantation. Specimens underwent demineralization and undemineralization processing for histological analysis. Demineralization was completed using 10% ethylenediaminetetraacetic acid in phosphate-buffered saline.

Colony-Forming Efficiency Assay

As part of *in vitro* testing, small subsets of the original bone marrow single cell suspensions were prepared for the determination of BMSC colony-forming efficiency (CFE) at the time of primary plating. For four of the animals, each of four 25-cm^2 flasks was plated with 1×10^5 nucleated bone marrow cells. These were incubated at 37°C in an atmosphere of 100% humidity and 5% CO_2. After 10 days, the flasks were rinsed with Hanks' balanced salt solution (Invitrogen), the cells fixed with absolute methanol, and the adherent BMSC colonies stained with saturated aqueous solution of methyl violet (Sigma). Fibroblast colonies of greater than 50 cells were counted under a stereomicroscope and the CFE, or the number of colonies per 1×10^5 nucleated bone marrow cells, was calculated.

Noninvasive Monitoring of Transplants Using Quantitative Computed Tomography (qCT)

From 2 weeks to 18 months after transplantation, the dogs individually underwent CT scanning in a GE CTI (GE Medical Systems, Milwaukee, WI) at an energy of 80 kVp/200 mA, a slice thickness 1 mm, and an in-slice resolution of 0.625 mm. The 2-week time points represented baseline information about the transplants, whereas the later time points were timed to closely match tissue sampling. Each scan included a Mindways Model 2 phantom (Mindways Software, South San Francisco, CA). The bone mineral density (BMD) of each image slice of each transplant was obtained using QCT Pro v. 2.0.3 (Mindways Software) on a Dell XPS R450 (Dell Computer Corp., Round Rock, TX). BMD values were expressed in terms of mg/cc of K_2HPO_4 in distilled water, in which a BMD of 0 corresponded to the density of distilled water alone (no additional K_2HPO_4) and a BMD of >0 corresponded to nonaerated biological tissue.

Each transplant was represented by 7 to 27 slices, each 0.488 mm thick and bounded by an oval-shaped region of interest (ROI). The software provided the ROIs, whose size and shape could be determined by the investigator. In each slice of a transplant, the ROI was adjusted to match the size and shape of the transplant, which was typically round in cross-section. Because the ROI and the transplant silhouette were well matched to each other, the ROI encompassed nearly the entire (>90%) area of the transplant at each slice. The BMD of the center seven slices of a transplant, encompassing the center 2.9 cm of the transplant, were pooled; the mean of these individual BMD values was used as the overall BMD value for the transplant. All BMD values were calculated by the primary investigator (M.H.M.), who was blinded to the bone scores of the transplants.

In Vivo *Noninvasive Ultrasound Testing of Transplants*

We used an experimental ultrasound system that is capable of generating a noninvasive, high-resolution ultrasound wave to the calvarium of the dog.[19–21] To transmit a signal to the calvarium, ultrasound transducers were designed in the frequency range of 0.5 MHz to 1 MHz. To obtain high-resolution signals associated with bone structure, the ultrasound signal was focused as a beam cylinder in a zone of ~0.5 to 1.0 mm in diameter and 2 to 3 mm in length. The transmitter was driven by pulse signals; these passed through bone and were then received by the receiver and amplifier unit and digitized at 25 MHz. The digitized waveform was then transferred to a personal computer (Dell Latitude, Austin, TX) for analysis. The control software was written using C++.

Reflect mode was used during the measurements, in which the waveform reflected from the bones' periosteal and endosteal surfaces was captured by the same transducer. The time delay Δt reflects the temporal difference between acquisition of the reference signal and the bone signal, and reflects in part the velocity difference between the bone specimen and the coupling gel. The ultrasound wave velocity V_b in trabecular bone was calculated using the time of flight method, in which the thickness of the specimen w and ultrasound wave velocity in water V_w are known.

$$V_b = \frac{V_w w}{w - V_w \Delta t}$$

The bone mechanical modulus, specifically Young's modulus, E, was then predicted by the relation

$$E = \mathrm{r} V_b^2$$

where r was the density of bone.

Ex Vivo *Mechanical Testing of Transplants*

A specially designed mechanical testing protocol was used to quantify bone strength in the control and experimental groups to characterize the differences in stiffness between BMSC-containing and BMSC-free transplants. From all six dogs, the entire calvarium, including transplant and surrounding intact normal calvarium, were harvested as a unit. Tissues were fixed in 4% paraformaldehyde for 24 hours, followed by storage in 1× phosphate-buffered saline (PBS) at −20°C until actual testing. Strips measuring ~5 mm in width were cut from the center of each specimen; these incorporated normal calvarium at each end and transplant material in between. The strips were further sectioned using a slow (100 rpm) speed saw (TechCut II; Allied High Tech Products, Inc., Rancho Dominguez, CA) into beam-shaped specimens measuring ~3 mm × 4 mm × 6.5 mm in size. The soft

tissues were operatively removed from the calvarium and transplant. Such specimens were taken from three sites: 1) the margin of the transplant, near its interface with the normal calvarium; 2) the center section of the transplant; and 3) the normal calvarium at the ends. Before actual testing, the specimens were thawed for 1 hour and then soaked in PBS for 1 hour. The mechanical testing for measuring the elastic (compressive) modulus was then conducted in ambient air (25°C, 20 to 40% relative humidity), with the specimens being loaded in displacement control using a standard servo-hydraulic testing machine (MTS 810; MTS Systems Corp., Eden Prairie, MN) with a loading rate 0.015 mm/second (to simulate quasi-static loading conditions). The elastic (Young's) modulus was then calculated from the load-displacement data obtained during such tests, with care being taken to eliminate the initial toe-in region. Three such measurements were made on each specimen. The operator was blinded to whether each specimen came from the experimental or control group. The data obtained were analyzed statistically using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA).

Results

Six dogs underwent bone marrow harvest, BMSC expansion, and BMSC autografting to calvarial defects. The first set of three dogs underwent calvarial bone biopsy at 2 to 3 months after transplantation and was sacrificed at 18 to 20 months. The second set of three dogs was sacrificed at 6 to 7 months. All animals healed their scalp incisions without problem, after both the transplantation operation and the biopsy procedure. Minute dura mater tears were created during a small number of the transplantation procedures and these were managed by immediate dural repair as well as postoperative antibiotic therapy. No cerebrospinal fluid leaks or infections were subsequently noted in any of the dogs' scalp operative sites. In cell culture, the dog BMSCs were fibroblastic in morphology. CFE, or concentration of BMSC colony-forming cells, of the first four dogs measured 81.5 ($\pm$12.6) per 10^5 nucleated cells from the distal femur.

Transplant Morphology

All calvaria exhibited good adherence of the overlying temporalis musculature to the transplant, at the time of both biopsy and transplant harvest. Examination of the transplants demonstrated a cohesive structure, with HA/TCP particles bound to each other throughout the transplant; no loose HA/TCP particles were noted in any transplant. Particles were found within the confines of the transplant, with no gross evidence of particle migration. Examination of the brain after calvarial removal showed the dura mater to be intact, and no particles were found deep to the dura. BMSC transplants were rigid on gross palpation and were firmly adhered to the adjacent normal calvarial margin. In contrast, BMSC-free transplants were not rigid and could be flexed with digital pressure while *in situ*. They exhibited a nonrigid binding to the surrounding normal calvarial edge.

Histologically, transplants without BMSCs contained aggregates of HA/TCP particles separated by fibrovascular tissue (Figure 2). Fibrovascular tissue also lay at the interface between the transplant and adjacent normal calvarium. In contrast, BMSC transplants contained abundant new lamellar cortical bone firmly attached to both particles and the adjacent calvarium (Figure 2). In transplants with more extensive bone (bone scores $\geq$3), bone was accompanied by hematopoietic tissue and occasional adipocytes, all of which were spatially associated with the HA/TCP particles. Adipocytes were seen frequently among BMSC transplants, as visualized in Figure 2, B and C. Very few adipocytes were seen in the BMSC-free transplants and were only seen in conjunction with new bone. Innervation of the transplants was not seen using standard histological techniques, including hematoxylin and eosin staining. There was abundant evidence of vascularization of both BMSC-free and BMSC transplants; gross examination of the transplants at the time of harvest suggested that vascularization was greater among the BMSC transplants.

Timing and Extent of Bone Formation

Among the first set of three dogs, both calvarial sides were biopsied at 2 to 3 months after transplantation. Bone scores among transplants on the control side ranged from 0 to 2.0 (mean, 1.33) and those transplants in defects filled with BMSCs had bone scores of 2.0 to 3.0 (mean, 2.33) (Figure 3). At the time of harvest at 18 to 20 months, bone scores ranged from 2.0 to 3.0 (mean, 2.33) among BMSC-free transplants and 3.7 to 4.0 (mean, 3.90) among BMSC-inclusive transplants ($P < 0.05$). The second set of three dogs, harvested at 6 to 7 months, had bone scores of 3.0 to 3.7 (mean, 3.23) among BMSC-free transplants and scores of 3.3 to 4.0 (mean, 3.77) among BMSC transplants (Figure 3). Comparison of all BMSC-free transplants with all BMSC transplants demonstrated significant differences in cumulative bone scores (mean, 2.30 versus 3.33; $P < 0.01$).

The first set of dogs demonstrated an increase in bone score between the biopsy specimens (2 to 3 months) and the harvest specimens (18 to 20 months), reflecting an increase in bone formation during the interval among both control and BMSC transplants. The second set of dogs had only one harvest time point; thus, the progress of bone formation could not be monitored as it had been among the first set of dogs. However, the second set of dogs had a higher control group mean bone score at 6 to 7 months than the first set of dogs had at the later time point of 18 to 20 months, reflecting better bone formation among this second set of dogs. In contrast to the trends seen among BMSC-free transplants, the BMSC-containing transplants demonstrated increasing bone formation with transplant age, regardless of which set of dogs was sampled (Figure 3). We attribute the better bone formation in BMSC transplants among the second set of dogs to our greater facility in handling these large volumes of cells and in placing these transplants in the animals.

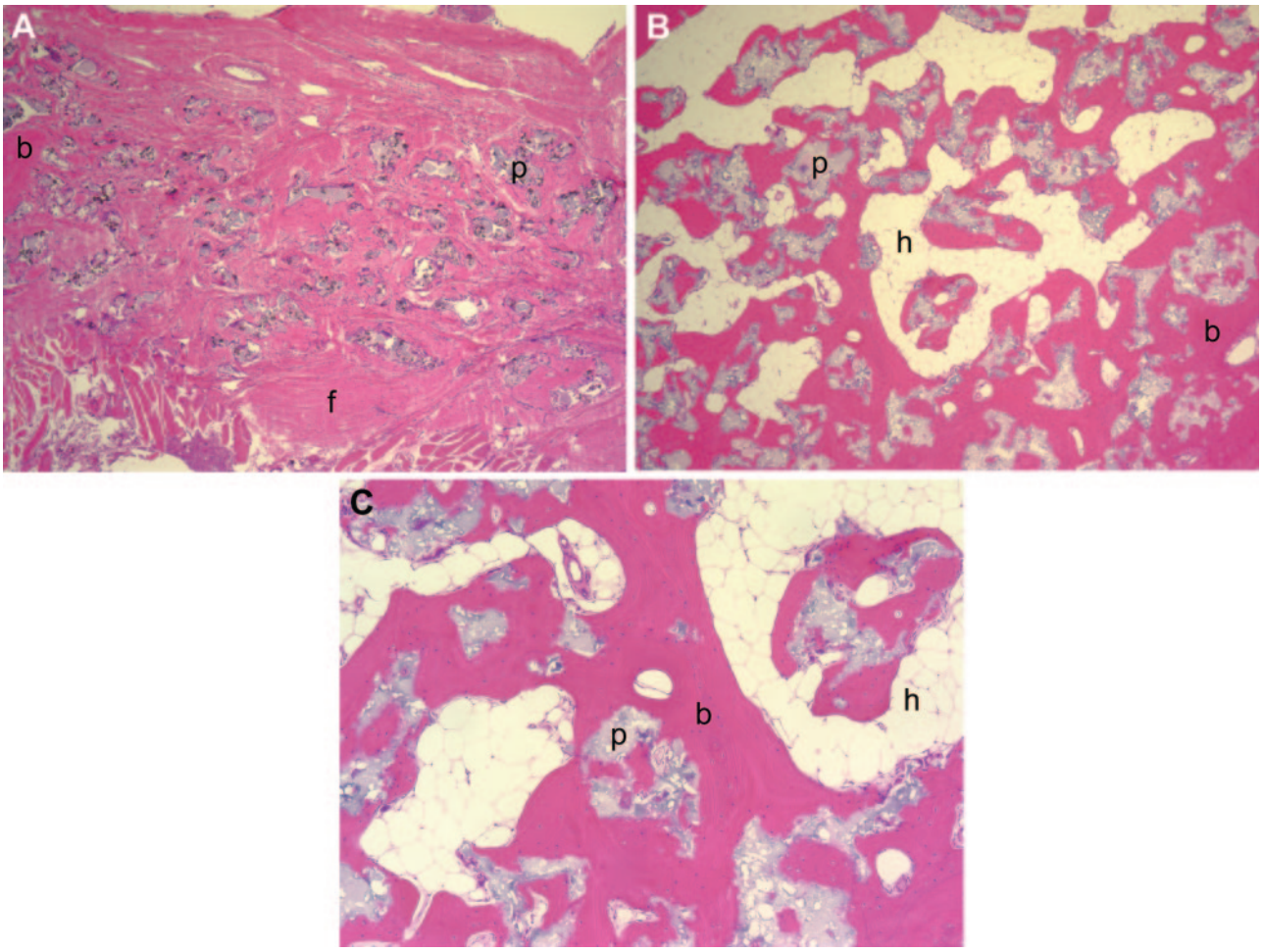

Figure 2. A: BMSC-free transplants 18 months postoperatively. Note extensive fibrovascular tissue with interspersed HA/TCP particles and only limited bone. **B:** BMSC-containing transplant 18 months postoperatively. Note extensive cortico-cancellous bone and hematopoietic adipose tissue. **C:** Higher power image of **B**. Note lamellar structure of bone, which is intimately associated with each particle. b, bone; f, fibrous connective tissue; p, particle, h, hematopoietic tissue.

Bone Union

The degree and distribution of bony union, or that union seen between the transplant and surrounding calvarial margin, mirrored the extent of bone formation (Figure 4). Bone union could only be evaluated among fully harvested transplants, not transplant biopsies, because biopsy specimens were obtained from the center of the transplants. Bone union ranged from 25 to 50% (mean, 35%) among 6- to 7-month-old control transplants, whereas it ranged from 50 to 100% (mean, 74.3%) among 6- to 7-month-old BMSC transplants (Figure 5). Among transplants harvested at 18 to 20 months, bone union ranged from 11 to 33% (mean, 20%) in the control transplants, whereas it ranged from 67 to 94% (mean, 81%) in the BMSC transplants ($P < 0.05$). Comparison of all BMSC-free transplants with all BMSC transplants demonstrated significant differences in bone union (mean, 27% versus 78%; $P < 0.01$) (Figure 5).

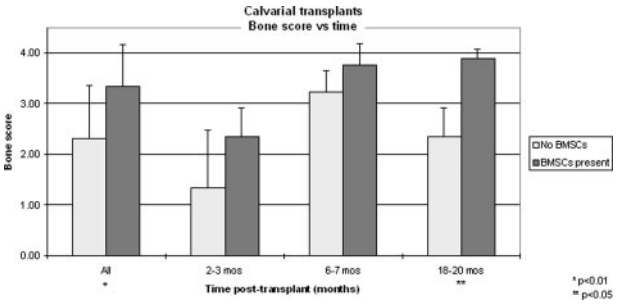

Figure 3. Bone score as a function of time and transplant type. "All" includes transplants at time of harvest (6 to 7 months and 18 to 20 months) and biopsy (2 to 3 months). Error bars represent SD.

qCT Density Data

CT images of the dog calvaria were obtained immediately after transplantation and coincident with the biopsies and calvarial harvests. Each image included a standard phantom for calibration purposes. Even without qCT analysis of the transplants, ossification of the BMSC-rich transplants and resorption of the BMSC-free transplants was evident by 6 months (Figure 6). The amount of bone formation within the transplants ranged from a bone score of 0 to a score of 4, and BMD values ranged from 443 to 838 mg/cc K_2HPO_4. The distribution of BMD relative to bone score is graphically represented in Figure 7.

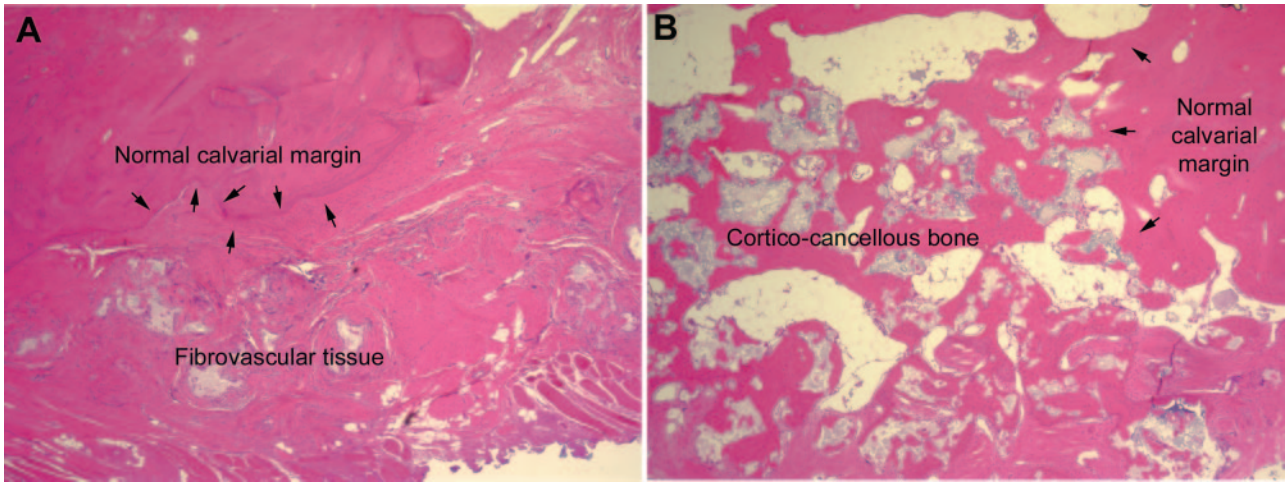

Figure 4. A: BMSC-free transplants 18 months postoperatively. Note extensive fibrovascular tissue, with little new bone forming a union with calvarium. **B:** BMSC-containing transplant 18 months postoperatively. Note appreciable zones of union between calvarium and new cortico-cancellous bone.

Seven transplant specimens showed no or poor bone formation (bone scores of 0, 1, or 2), whereas the remaining 11 transplant specimens formed appreciable amounts of bone (bone scores of 3 or 4). A significant positive trend in BMD was detected ($P < 0.0001$) as a function of clinical bone score. The estimated relationship is given as BMD = 100.6 × (bone score) + 369.3 with correlation coefficient (r) = 0.8174.

Increasing bone scores were associated with increasing BMD. Transplant specimens with a bone score of 0 to 2 were associated with BMDs of 400 to 631, whereas those with a bone score of 3 to 4 were associated with BMDs of 580 to 838. BMD served to distinguish between poor (bone score <3) bone formation and appreciable (bone score ≥3) bone formation among transplants with or without BMSCs (Figure 8). The BMD among BMSC-free transplants with either poor or appreciable bone formation averaged 501 and 681 mg/cc K_2HPO_4, respectively ($P < 0.05$), whereas the BMD among BMSC transplants with either poor or appreciable bone formation averaged 589 and 763, respectively ($P < 0.005$). When BMSC-free and BMSC-inclusive transplants were pooled, the mean BMD of poor bone formers and good bone formers was 526 and 733, respectively ($P < 0.0005$).

If a BMD of 600 is used as a threshold to distinguish poor (bone score <3) bone formation and appreciable (bone score ≥3) bone formation (Mankani MH, Kuznetsov SA, Robey PG, unpublished data), then 15 of the 18 specimens corroborated this relationship (Figure 7). The three exceptions included two transplants with bone score of 2 and BMD of 619 and 631, respectively, and one transplant with bone score of 3 and BMD of 580.

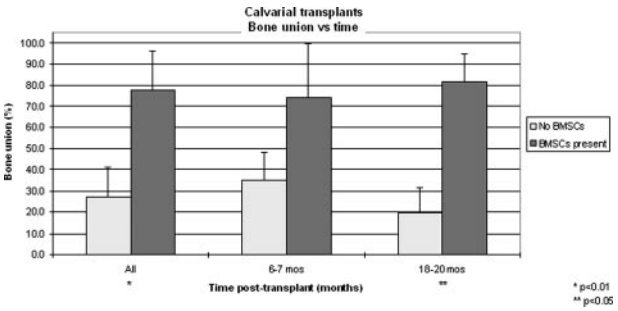

Figure 5. The extent of bone union as a function of time and transplant type. The data includes only transplants at time of harvest. Error bars represent SD.

In Vivo *Ultrasound Testing of Transplants*

Transplants from three of the animals underwent ultrasound testing before sacrifice. In each of the animals, there was a slight but not statistically significant difference in calculated elastic modulus *E* between the BMSC-containing transplant and the transplants that were free of BMSCs (Figure 9).

Ex Vivo *Mechanical Testing of Transplants*

Transplants from all six animals underwent mechanical testing. In each of the animals, the BMSC-containing transplant was found to have both a higher bone score and elastic modulus than the control tissue from the same animal. Bone scores among BMSC-free and BMSC-containing tissues were 2.30 and 3.33, respectively ($P < 0.05$), whereas the overall Young's modulus at the center of the same transplants was 0.24 and 0.59 GPa, respectively ($P < 0.001$) (Figure 10). The overall modulus of the tissue at the margins of the transplants was 0.24 and 0.90 GPa, respectively ($P < 0.005$). In contrast, the modulus for normal calvarial bone among the six dogs averaged 1.85 (±0.29) GPa.

Discussion

BMSCs are arousing increasing interest as potential sources for cell-based implants to reconstruct hard tissue defects. They have been shown to repair defects in the mouse calvarium and the rat femur, and they can serve as a reliable cell source for the creation of vascularized bone flaps.[15,22,23] Thus far, such defects and their cor-

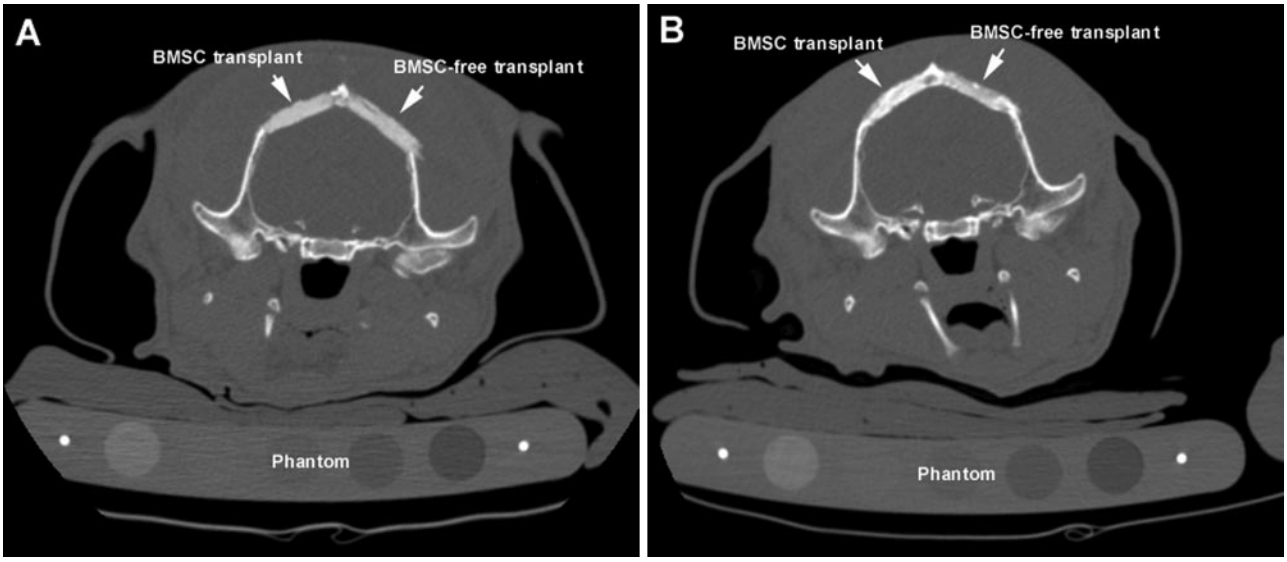

Figure 6. A: CT image of dog head immediately after transplantation. qCT phantom is included with all examinations to provide density data. **B:** Six months after transplantation, the BMSC-containing transplant exhibits a greater density consistent with ossification, whereas the BMSC-free transplant exhibits a lucency consistent with HA/TCP particle resorption.

responding transplants have been of limited size and are far smaller than the transplants that would conceivably be used to close calvarial or long bone defects in patients. Clinically relevant transplants would be expected to be 100 to 500 times the volume of transplants generated for rodents; this larger size creates its own challenges. Such transplants require a corresponding increase in the number of cultured BMSCs, and they require an even more hospitable recipient bed to provide sufficient perfusion and vascularization. The challenges posed by scaling up the size of BMSC transplants served as the motivation for this study, which sought to 1) demonstrate the feasibility of closing clinically relevant critical-size calvarial defects, 2) demonstrate the practicality of scaling up BMSC culture and transplant preparation procedures, 3) complete noninvasive evaluations of the extent of new bone formation, and 4) assess the mechanical properties of large-scale BMSC transplants.

In this study, we transplanted culture-expanded autologous dog BMSCs into sizable calvarial defects, defects that were far larger than would be expected to spontaneously close. The cells had been combined with an osteoconductive ceramic, HA/TCP, just before transplantation. Each dog also received a HA/TCP transplant devoid of BMSCs in an identical contralateral calvarial defect. These BMSC-free HA/TCP transplants are a good control because they are commonly used by craniofacial surgeons to close bone defects and represent the standard of care. From periods of 2 to 20 months after transplantation, dogs were evaluated using histology, CT scanning, and ultrasound. The BMSC-containing transplants formed bone faster and more extensively than transplants containing only the HA/TCP osteoconductive matrix. The newly formed bone developed a union with the adjacent margin of calvarium, and the extent of bone union was significantly greater among the BMSC-containing transplants. BMSC-containing transplants maintained their cohesiveness, such that particles did not migrate from the transplant into the surrounding tissues. The transplants maintained their shape and size despite the absence of mechanical stimuli that promote remodeling and exhibited signs of continued new bone formation as late as 20 months. In contrast, BMSC-free transplants demonstrated zones of particle resorption and migration. Noninvasive testing of the transplants with qCT demonstrated that BMD increased in proportion to bone score, and that, with infrequent exception, a BMD of 600 served

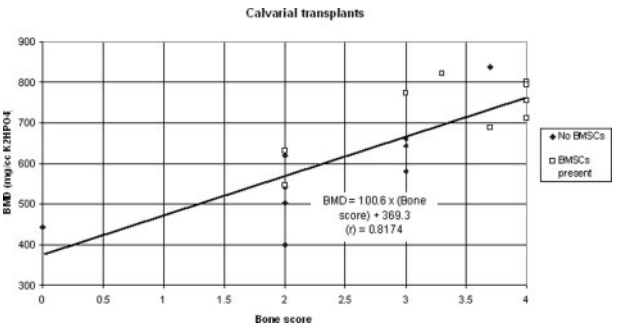

Figure 7. Scatter plot of bone score versus BMD for transplants at time of biopsy or harvest.

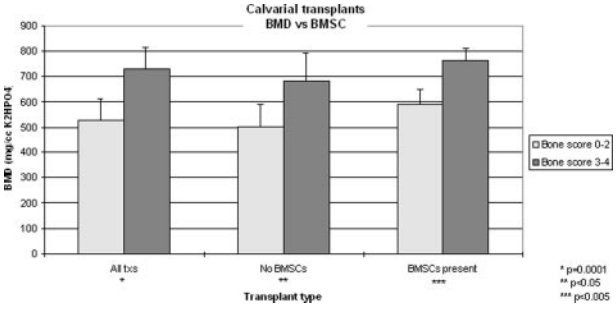

Figure 8. BMD as a function of transplant type, distinguished among transplants with poor bone formation (bone score 0 to 2) or good bone formation (bone score 3 to 4). Error bars represent SD.

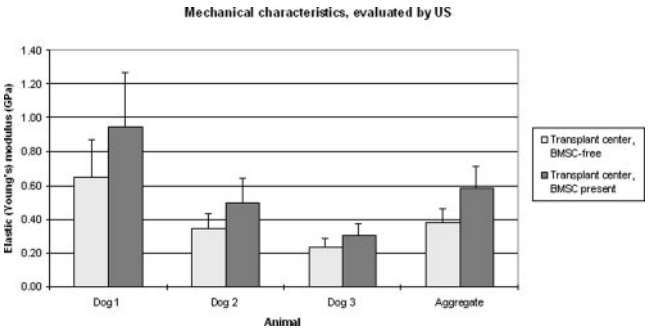

Figure 9. Elastic (Young's) modulus, as determined by noninvasive ultrasound evaluation. Elastic modulus *E* is uniformly greater among transplants with BMSCs. Error bars represent SD.

as a reliable threshold distinguishing poor bone formation from significant bone formation. *Ex vivo* mechanical testing of the transplants demonstrated that those with BMSCs had mechanical properties more closely matching that of normal bone than the BMSC-free transplants; additionally, such differences could be detected noninvasively with ultrasound.

This study was designed to closely prepare for a clinical trial. Dogs were specifically selected as the research subject because of their well-established history in bone graft studies, their size, and the characteristics of their BMSCs. Surgically induced calvarial defects in dogs are a well-established model for evaluating bone regeneration as well as the success of bone-grafting materials.[18,24,25] The diameter of the calvarial defects created in this study is far larger than that which would be expected to spontaneously close. The overall size of the animals was also important, because it allowed for the nondebilitating harvest of bone marrow in amounts comparable to that expected clinically. Animal size also permitted CT scanning, qCT analysis, and ultrasound testing, which closely parallels that conducted in patients, and the size of the transplants was substantial enough to permit meaningful mechanical studies of the mature transplants. Dog BMSCs have characteristics that parallel those of human BMSCs. Their CFE values have been reported to range from 2 to 10 per 10^5 nucleated marrow cells,[26,27] not greatly different from the CFE of 81 per 10^5 among the dogs in this study and close to the CFE of 21

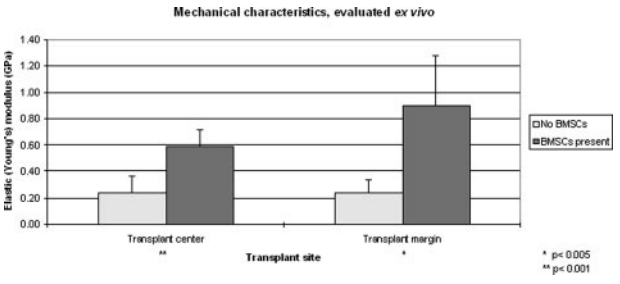

Figure 10. Elastic (Young's) modulus of transplants, determined during *ex vivo* testing, at both their centers as well their margins. BMSC-containing transplants had uniformly higher *E* than their BMSC-free counterparts, whether measured at the center or margins of the transplants. A specific zone in one transplant (such as the center or margin of the transplant) is being compared to its counterpart on the contralateral transplant. Error bars represent SD.

to 77 per 10^5 of normal human BMSCs.[28,29] Transplants of dog BMSCs combined with HA/TCP particles form cortico-cancellous bone when placed in immunocompromised mice, but transplants of dog BMSCs combined with bovine collagen sponges fail to form bone (Mankani MH, Kuznetsov SA, Robey PG, unpublished data); these findings closely match our experience with human BMSCs and are quite distinct from the characteristics of other species' BMSCs.[8] As would be planned clinically, autologous BMSCs were used in this study. Our experience with human and mouse BMSC transplants placed in mice suggest that the new bone found in these transplants originates from the implanted BMSCs and the hematopoietic tissues originate from the recipient.[8,15,23] Although the transplanted BMSCs in this study were not labeled and therefore could not be tracked after transplantation, the profound differences in bone formation between BMSC-free and BMSC-containing transplants suggests that BMSCs were responsible for bone formation where they were placed.

A semiquantitative scale of bone formation was used in this study. When the bone scores reported on this scale have been compared to histomorphometric measurements of tissue sections, a correlation was previously observed between the bone score and the square root of the fraction of bone area to total transplant area (B/T) ($r = 0.973$).[30] Bone scores of 0, 1, 2, 3, and 4 correlated with fractional bone areas of 0%, 1%, 3%, 8%, and 14%, respectively. The poor bone formation described in this study would be characterized by a fractional bone area of $\leq$3%, whereas appreciable bone formation correlated with a fractional bone area of $\geq$8%. Thus, the differences in bone score noted between BMSC-free and BMSC-containing transplants parallels significant differences in transplant fractional bone area B/T.

Transfer of the BMSC transplantation techniques to patients requires the development of a noninvasive method for evaluating the extent of new bone formation among these transplants. HA/TCP has a high density that is comparable to that of cortical bone. Use of plain radiographs is insufficient because they fail to distinguish HA/TCP transplants that have no associated bone formation from those that do (Mankani MH, Kuznetsov SA, Robey PG, unpublished data). As an alternative to radiographs, we have previously demonstrated that the utilization of qCT techniques could be adapted to BMSC-HA/TCP transplants to describe the extent of bone formation within the transplants.[31] Among transplants of human BMSCs in mice, we noted that a BMD of 600 mg/cc K_2HPO_4 serves to distinguish poor bone formation (bone scores of 0 to 2) from appreciable bone formation (bone scores of 3 or 4). In the present dog study, we note that all but 3 of 18 specimens in this large animal model corroborate findings from our mouse study, regardless of whether the transplants used BMSCs and regardless of their harvest date. Care was also taken to specifically use a clinical CT scanner rather than a unit used for investigational purposes. Although a research scanner such as MicroCT would undoubtedly have provided much better resolution and a finer slice thickness of these dog transplants, and would therefore have described the bone

architecture better, it was important to us to demonstrate the feasibility of using qCT with clinically appropriate equipment and settings.

Transcutaneous ultrasound testing of bone is a relatively new and novel method for assessing the mechanical characteristics of this tissue.[19,20,32] This represents the first report of its use to estimate the stiffness of bone-containing calcium phosphate transplants. Although this technique discerned a difference in modulus between the transplant types, the Young's modulus values only approximately coincided with those of the gold standard study, *ex vivo* mechanical testing. We attribute the modest correlation with *ex vivo* testing to the heterogeneity of the transplants and to the likelihood that the areas sampled by ultrasound did not necessarily coincide with the areas evaluated *ex vivo*.

In summary, we have used an established bone defect model to demonstrate the feasibility of using large volume autologous BMSC transplants to close critical-sized calvarial defects, and we have confirmed that the extent of bone formation can be reliably estimated using noninvasive techniques. The success of such large transplants increases the practicality of transplanting culture-expanded BMSCs in patients.

Acknowledgments

We thank Zimmer, Inc. (Warsaw, IN) for the gift of HA/TCP; Mindways Software (San Francisco, CA); Dr. Chris Cann for technical assistance; and Dr. Nilo Avila, Mr. Dennis Johnson, Mr. David Williams, and Mr. Ronald Norman (Department of Radiology, Clinical Center, National Institutes of Health, Bethesda, MD) for assistance with the imaging.

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